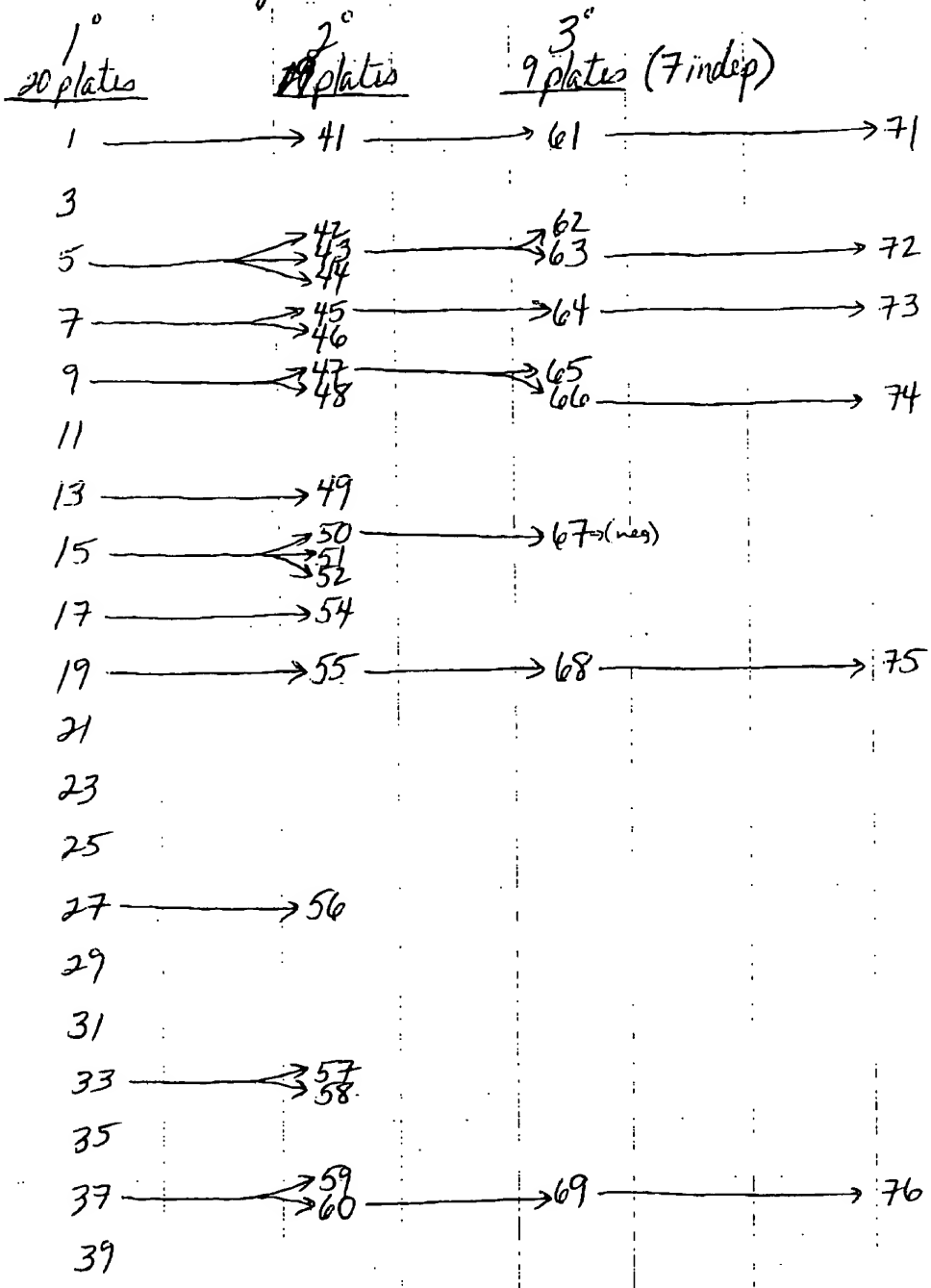
Exhibit 9

Lab Notebook Pages of
Dr. Susan L. Acton

Successive Rounds of screening to clone the murine SR-BI from a 3T3-L1 Adipocyte pcDNAI Library



Probe: 500-600 bp BamHI fragment (5') of huSR-BI in pcDNAI

3T3-L1 Adipocyte Library

pCDNAI in MC1061/P3

G. Balding electroporated + plated prep, then scraped plates + froze. This is what she gave me. (3 vials)

Use vial 2

Scrape a little + thaw, dilute 1:100, then 1:50 and plate 300 µl on a 10 cm plate

She did 20 plates of 30,000 colonies (must have been very small colonies)

Note that the library is non-directional + is in the BstX I sites in pcDNAI

XJ58 Screen 3T3-L1 Adipocyte Library for murine SR-BI

Purpose: Clone the murine SR-BI for further analysis

Plate Library

Pour 100mm LB Amp/Tet (15/8 μg/ml) Plates
Let dry 48 hrs r.t.

Scrape some bugs from frozen tube 2 (from G. Baldini Lodish lab) of 3T3-L1 library in MC1061/P3 pGDNA1 expression)
Thaw scraped bugs, dilute 1:100, then 1:17 and plate 50μl

2 μl undil bugs tube #2
198 μl LB

200 μl of 1:100
↳ 118 μl of 1:100
1.882 ml of LB

2 ml of 1:17
↳ plate 50μl/100mm plate

Incubate plates 14 hrs (6pm-8am) 37°C
Transfer plates to 4°C 1hr

Make Lifts

① Carefully lay down sterile Nitrocellulose Millipore HATF prenumbered, number side down onto bugs
② When wet, poke 3 holes thru membrane and plate
③ Lift filter off plate with blunt end forceps

~~Lap~~

④ Lay bug side up onto fresh LB A/T plate
⑤ Lay another filter (pre-numbered) numby-side down onto bugs on filter to make sandwich
⑥ Poke holes thru duplicate filter to match holes in original filter
⑦ let bugs grow 3 hrs 37°C between filters
⑧ Let bugs regrow on original ~~filter~~ plate 3 hrs - then store wrapped in parafilm 4°C.
⑨ As in Maniatis, (1.98) lyse colonies
Set up 4 trays with whatman 3MM Paper soaked with (in order)
① 10% SDS
② Denaturing soln (0.5N NaOH 1.5M NaCl)
③ Neutralizing soln (1.5M NaCl, 0.5M Tris-Cl pH 7.4)
④ 2X SSC

a) Peel nitrocellulose sandwich from gel & place on SDS-impregnated 3MM paper 2-3 min
b) Wipe off excess with edge of tray & transfer to denat. tray 5 min
c) Transfer to Neutral tray 5 min.
d) Transfer to 2X SSC tray ~~5 min~~ but separate sandwich first - 5 min
i) Transfer to dry 3MM paper colony side up Dry at least 30 min r.t.

⑩ Sandwich filters between 2 sheets of dry 3MM paper Fix by baking, in 80°C in vacuum oven
⑪ Store filters under vacuum no heat

X158 cont'd

Make SR-BI probe

Digest #		10X Ac BSA	H_2O	10X NEB Buffer	enzyme
① phaSRIII (1.03 µg/µl)	10µl	5µl	28µl	5µl #2	PstI 1µl / HindIII 1µl ↓
② pcDNA1 (1 µg/µl)	10µl	5µl	28µl	5µl #2	
③ phaSRIII	10µl	5µl	28µl	5µl (BamHI buffer)	BamHI 2µl ↓
④ pcDNA1	10µl	5µl	28µl	5µl (BamHI buffer)	

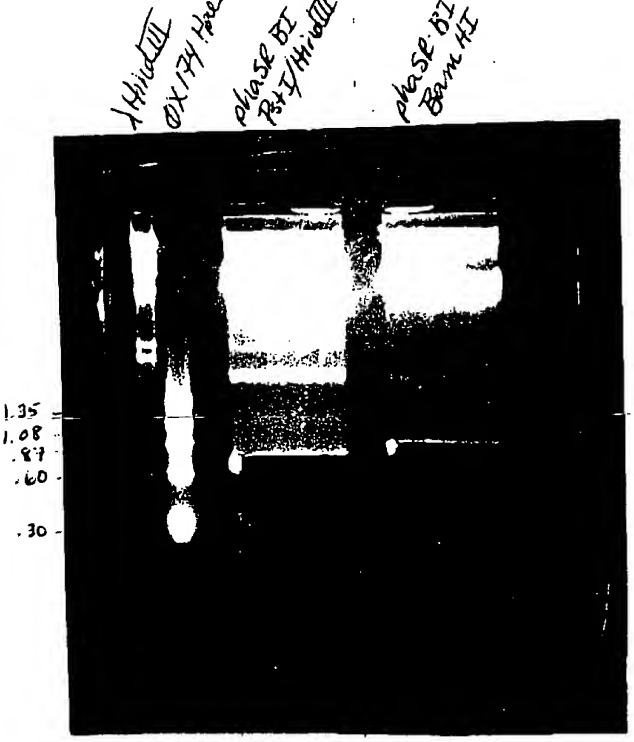
Incubate 37°C 2hr 2:10-4:10 freeze digest
Ran mini-gel; all digests looked good.
Clean up digests ① + ③

Phenol extract (50µl)
Back extract w/ 50µl TE
Precip DNA in 100µl
with 10µl 3M NaOAc
260µl 100% EtOH
-80°C 20 min
5min 20 min 4°C
Wash w/ 70% EtOH (cold)
Spin Vac Dry
Resuspend in 18µl TE
Add 6µl 10X blue juice
Heat 68°C 10 min

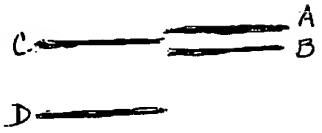
Run on 1% Low melt (Seaplaque) TAE gel 4°C overnight 6V turned up to 20V at noon Cut out band at 6 pm

λ HindIII ØX174 AseIII Digest ① 4 lanes Digest ③ 4 lanes

X458 cont'd



Probes



For screening lifts, use probe B

Label Probe

① Melt probe B, take 10µl + add to 10µl H_2O. Label as probe B diluted 1:1

② Combine 5µl diluted probe B
5µl primers (from Amersham kit 9mers)
Boil 5'
spin down liquid

③ Add 10µl labeling buffer (kit)
23µl H_2O
2µl enzyme
5µl $\alpha^{32}P$ dCTP
Mix by pipetting gently
Spin
Put in 37°C warm room ~~45 min~~ 2hr 2pm - 4pm

X158 cont'd

Check incorporation

Combine
20 µl salmon sperm DNA
<1 µl label rxn
180 µl 5% cold TCA

Spin 10' 4°C
Remove 100 µl & count as top
Count remainder as bottom

% incorp = $\frac{\text{bottom} - \text{top}}{\text{bottom} + \text{top}} \times 100\% = 22\%$

% incorp = 22%

Clean up probe:
Add 5 µl LPA to rxn
25 µl 7.5 M NH_4 Acetate
125 µl 100% EtOH (cold)
Put at -80°C 20 min
Spin 20 min 4°C
Rinse w/ cold 70% EtOH
Speed-Vac dry
Resuspend in 30 µl TE
Add to prehyb soln containing filters
Hybridize 50°C overnight

X158 cont'd

Prehyb - 50°C

500 mM PB, 1mM EDTA, 7% SDS, 1% BSA, 100 µg/ml salmon sperm DNA (boiled prior to adding)

For 60 mls: 30 mls 1M PB ✓
120 µl 500 mM EDTA ✓
21 mls 20% SDS ✓
0.6g BSA ✓
0.6 ml salmon sperm DNA ✓
8.28 ml H_2O ✓

Hyb - 50°C in prehyb soln + probe

Washes -

Wash blot 2X fast w/300mM PB r.t.
wash " 1X 10' r.t.
Wash " 2X 10' Soln A (300mM) 54°C
Wash " " " B (300mM) 54°C

Put down on film - 48 hrs r.t.
a) Let filters air dry on whatman
b) Arrange on old piece film covered with saran wrap
c) ~~Cover~~ & Tape down filters & cover w/saran wrap
d) Put markers (from Stratagene ruler) down to orient film w/ filters

Results:

> 100 small dots which are on duplicate lifts. Background is low. Looks good!

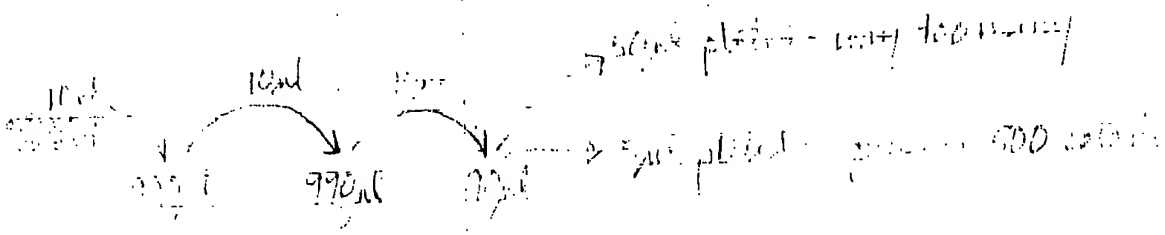
IX158 cont'd

Secondary Lifts

A. Picked 19 positive dots / negative control from primary lifts

B. Used pasteur pipets, large end to pick plugs from plates into 0.5 ml LB + Amp / Tet Overnight 37°C

C. Make dilutions



D. Plate bugs
First time plated way too many so replated ~13 hrs 37°C

E. Transfer plates to cold room 1 hr 4°C

Lifts

① Carefully lay down sterile HATF nitrocellulose Millipore prenumbered, number side down onto bugs

② When wet poke 3 holes thru membrane and plate

③ Lift filter off plate with blunt end forceps

④ Lay bug side up onto fresh LB A/T plate (LB Kan for 54-60)

⑤ Lay another filter (labeled B) number side down onto bugs on filter to make a sandwich

⑥ Poke holes thru duplicate filter to match holes in original filter

⑦ Let bugs grow 3 hrs 37°C between filters

⑧ Let bugs grow on original plate 3 hrs then store wrapped in parafilm 4°C

⑨ As in Maniatis (1.98) Lyse colonies
Set up 4 trays with Whatman 3MM paper soaked with

3 min	① 10% SDS
5 min	② Denaturing soln (0.5N NaOH, 1.5M NaCl)
5 min	③ Neutralizing soln (1.5M NaCl, 0.5M Tris Cl, pH 7.4)
5 min	④ 2X SSC

a) Peel nitroc. sandwich from gel + place on SDS-impreg. 3MM paper
b) Wipe off excess with edge of tray + transfer to denat. tray 5 min
c) transfer to neutral tray 5 min
d) Transfer to 2X SSC tray after separating filters
e) transfer to dry 3MM paper colony side up. Dry at least 30 min r.t.

⑩ Sandwich filters between sheets of dry 3MM paper. Fix by baking 1 hr 80°C in vacuum oven

⑪ Store filters under vacuum no heat

Label probe (5' BamHI fragment of haSR-BI) as for 1° lifts

[illegible] 3 ID:CERENKOV PRESET TIME: [illegible] TUI [illegible]
SAMPLE REPEAT: 1 CYCLE REPEAT: [illegible] SCR:N RS232:[illegible]
[illegible]
CHANNEL 1 [illegible] 1000 [illegible] SIGMA: 2.00 BKG [illegible] [illegible] BKG 2SIG: 0.00 LSR:
CHANNEL 2-LL:600 UL:1000 2SIGMA: 2.00 BKG SUB: [illegible] BKG 2SIG: 0.00 LSR:
DATA CALC: CPM, UNKNOWN REPLICATES: 1 NORM FACTOR:01.00000
HALF LIFE(DAYS):N

SAM	CPM1	CPM2	TIME
1	[illegible]36.54	757.00	1.00
2	[illegible].97	324.00	1.00

36% incorporation

[illegible] ~~[illegible]~~ 6 ok

Wash blots - Note: May have accidentally used 600mM PB final rather than 300mM

① Wash blot 1X fast w/ 300 mM PB r.t.
② Wash blot 1X 60' r.t. 300 mM PB
③ Wash 2X 54°C soln A 300 mM 10' each

Wash A: 300 mM PB, 5% SDS, 0.5% BSA, 1mM EDTA

For 500 ml: 300 ml 0.5M PB
25 g SDS
2.5 g BSA
1 ml 500 mM EDTA
H_2O to 500 ml

④ Wash 2X 54°C soln B (300 mM PB) 10' each

Wash B: 300 mM PB, 1% SDS, 1mM EDTA

300 ml 0.5M PB
15 ml 20% SDS
1 ml 500 mM EDTA
H_2O to 500 ml

Dry & expose to film

① Let filters dry on whatman paper
② Use old piece of film as support
wrap it in saran wrap
put filters on it & tape down
put stratagene ruler pieces on it to line up 1° & 2° filters
cover with saran wrap
③ Expose to film 2 days

Results: 2° lifts don't look as good as 1° - should not have amplified plug pick from 1°. I think high background may be due to accidental use of 600 mM PB rather than 300 mM PB.

Still there seem to be some definite positives so picked them for 3°.

X158 cont'd

3° Lifts

A. Picked 9 positive dots (#61-69), negative control (#70) from secondary lifts

B. Used sterile pasteur pipets, large end to pick plugs into 0.5 ml LB. Vortexed well.

LB
plug + 500 µl

		results
10 µl + 990 µl	→ plate 50 µl	way too many
10 µl + 990 µl	→ plate 50 µl	way too many
100 µl + 900 µl	→ plate 50 µl	just about right ~200/plate

Grow ~14 hrs 37°C

C. Transfered highest dilution plate to 4°C for lifts

D. Performed lifts as before

Made mistake of reincubating master plates for ~48 hrs rather than ~6-8 hrs. Plates dried somewhat and colonies grew big

E. Label probe as before

TUE

	CPM2	CPM2	TIME
1	137662.97	244.00	1.00
2	175257.98		1.00

37.5% incorporated

Prehyb } as before 30°C, 300mM PB
Hyb }

Wash 3° as before - 54°C, 300mM
...on Put down on film - w/screen

X160

Plasmid midiprep

preps: 6 single isolates from 3° screen #71-76

Day 1

1. Pick a single colony into 25 mls LB Amp/Tet and grow overnight shaking 37°C.

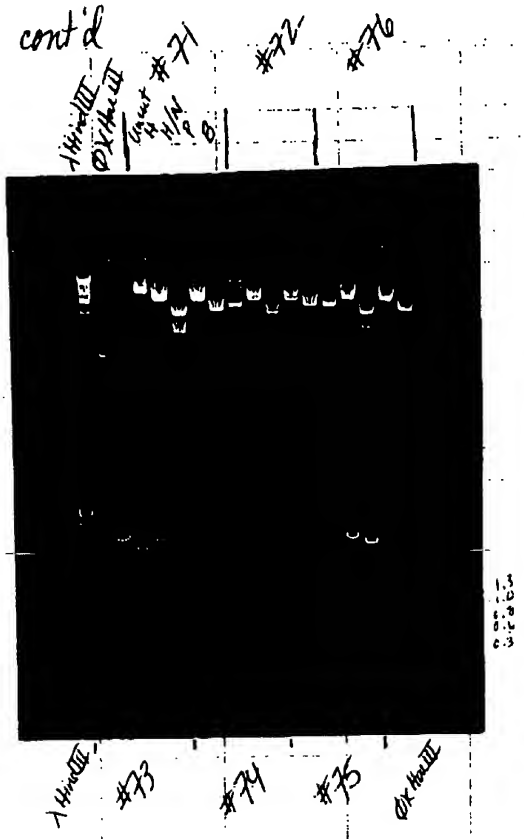
Day 2

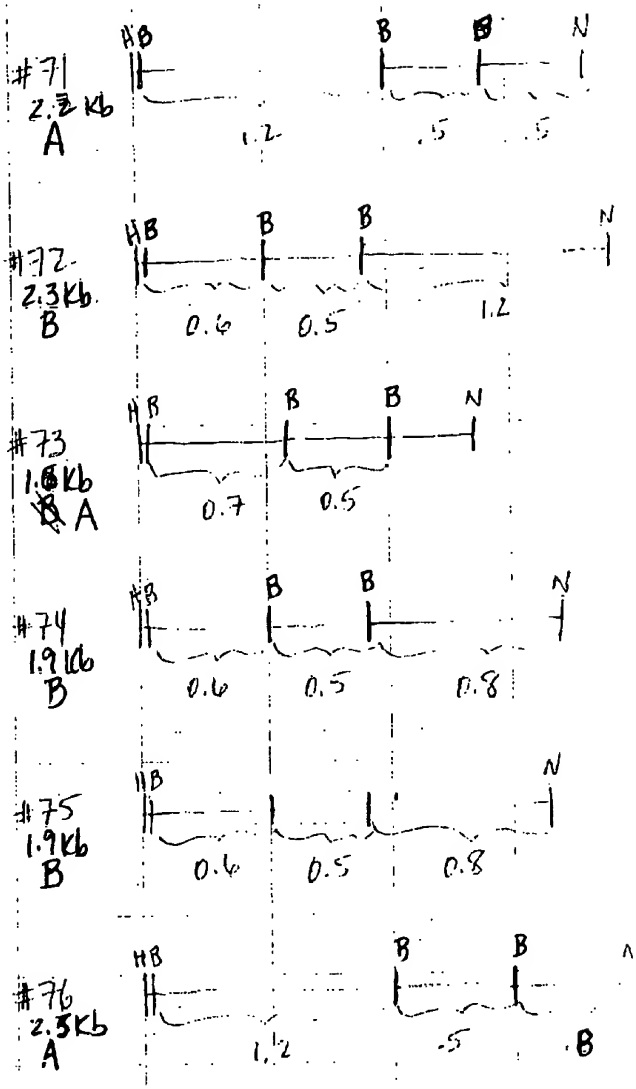
2. Take 400 µl out into freezer vial, add 100 µl glycerol and freeze at -150C.
3. Transfer ~~remainder~~ to Falcon 2059 15 ml tube on ice.
4. Spin ~~rest~~ in SS-34, 9000 rpm, 2 min 4C. Add remaining half.
5. Dry pellet as much as possible.
6. Resuspend pellet in 500 µl ice cold solution I by vigorous vortexing.
7. Add 1 ml fresh solution II (0.2 N NaOH, 1% SDS)
   for 100 ml: 1 ml 2 N NaOH
   0.5 ml 20% SDS
   8.5 mls ddH20
   Swirl gently until clear. Do not vortex. Leave on ice 10 min.
8. Add750 µl solution III (ice-cold). Close tube and mix contents by shaking vigorously several times. Store on ice 5'. A flocculent white precipitate should form.
9. Centrifuge 15', 4°C, 9000 rpm.
10. Recover supe and add equal volume of phenol:chloroform. Mix by vortexing.
11. Spin 9000 rpm, 5'.
12. Add 2 volumes, ethanol r.t., vortex, let stand 5'.
    Spin in SS-34 rotor for 15' 9,000 rpm.
13. Carefully aspirate off all liquid (want it dry) and rinse once in cold 70% EtOH. Aspirate again getting any extraneous drops. Airdry 10' minutes.
14. Redissolve in 100 µl TE ~~plus DNAse-free RNAse (20 µg/ml). Vortex briefly. Incubate 37C, 0.5 - 2 hr. Transfer to sterile eppendorf.~~ Refrig 4°C o.N.
15. ~~Quantitate by dilutions onto EtBr plate.~~
    Add DNAse free RNAse to final 10µg/ml

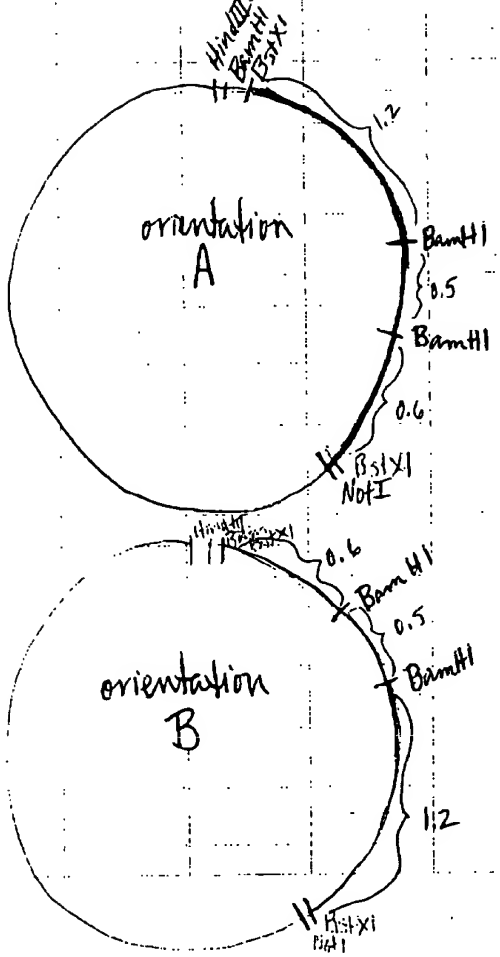
X160 cont'd Digests of Midi-preps

Digest #	DNA	10X AcBSA ml	NEB Buffer 10X	H_2O	Inl each enzymes	DNA Fragment
1	#71 - 2 µl	2 µl	2 · 2µl	14 µl	–	0.8 kb
2			2	13	HindIII	
3			2	12	HindIII / NotI	
4			2	13	PstI	
5			2	13	BamHI	
6	#72					
7						
8						
9						
10						
11	#73					
12						
13						
14						
15						
16	#74					
17						
18						
19						
20						
21	#75					
22						
23						
24						
25						
26	#76					
27						
28						
29						
30						
	37°C	11:45am - 2:45				

X160 cont'd







Note that orientation B was found to be correct after sequencing of #74 + #76

X1161

DEAE dextran transfections of COS M6 cells

materials:

1. 35 mm dishes
2. DMEM with 10% FBS
3. Chloroquine (40 mM in CMF PBS, sterile filtered)
4. DNA
5. CMF PBS
6. DEAE-dextran (10 mg/ml in CMF PBS (autoclaved))
7. DMSO
8. cPBS
9. sterile tips

method:

day 0 (set up cells)

Set COS M6 cells in 100 mm dishes

(Set 1 confluent T75 into 2.25 100 mm dishes; or 1.5 x 10^6 cells/dish in 10 ml DMEM with 10% FBS

day 1 (transfect)

1. In sterile polypropylene tubes prepare for each dish add (in order):
   a) add CMF PBS to 1.9 ml
   b) DNA - 18 µg/dish
   c) 100 µl of 10 mg/ml DEAE-dextran, vortex well (7 seconds)

tube	# plates	DNA			CMF PBS	10 mg/ml DEAE-dextran
1	1	pheSP-BI (1.	1.03 mg/ml	9.7 µl	1.9 ml	100 µl
2	1	#74 (7/22/94)	1.94 mg/ml	5.2 µl	1.9 ml	100 µl
3	1	#76 (7/22/94)	0.85 mg/ml	11.8 µl	1.9 ml	100 µl
4						

2. Rinse cells with 10 mls CMF PBS (37C). Aspirate PBS.
3. Add transfection cocktail from step 1 and distribute evenly. Incubate 30 minutes, distributing liquid every 10 min.
4. Add 8 ml DMEM 10% FBS + 80 µM chloroquine and incubate 37C 2.5 hrs.
5. Aspirate off medium and replace with 5 ml 10% DMSO in DMEM 10% FBS for 2.5 min.
6. Aspirate off and wash once with 10 mls cPBS.
7. Refeed with 10 ml warm DMEM 10% FBS/dish. Incubate overnight.

X161 cont'd

Lipoprotein 4°C Binding Assay

materials:
1. 6-well dishes
2. DMEM with 10% FBS
3. iodinated and unlabeled lipoprotein
4. Tris-HCl wash buffer
5. Tris-HCl BSA wash buffer
6. 0.1 N NaOH
7. 10 x 75 tubes

method:

day 0: Set up cells

Set transfected COS in 6-well dishes at 1 x 10^6 cells/well in 3 ml/well DMEM + 10% FBS + 1 mM Nabutyrate.

day 1: Binding assay

1. Cool cells down on ice for 30 minutes.
2. Refeed cells labeled ligand 1 ml/well (Hams + HEPES + ~~10% FBS~~ 5% NCLPDS).
   2 Hot - ^{125}I-lipoprotein: 10 µg/ml ^{125}I AcLDL (#61 10.4 mg/ml) LDL #161B (39 mg/ml)
   1 Hot + cold: + 50 µg/ml M BSA #230 DR (3.2 mg/ml)
   Incubate 2 hrs 4°C (in cold room) on shaker.
3. Wash cells (1 ml each)
   3X fast with BSA wash buffer
   2X 5 min with BSA wash buffer
   2X fast with Tris wash buffer
4. Add 1.0 ml 0.1 N NaOH. Leave 20 min r.t. on shaker
5. Remove 50 µl to 10 x 75 tube and freeze for Lowry.
6. Count 500 µl of rest of sample.
7. Count 10 µl of medium + label for specific activity.

For 14.5 mls hot need: 14.5 mls Hams + HEPES + 5% NCLPDS + 145 µl ^{125}I-AcLDL (#61)

5 mls + 51.3 µl LDL #161B

3 mls + 375 µl M-BSA #230

#74

-	+ LDL	+ M-BSA
1	3	5
2	4	

#76

6	8	10
7	9	

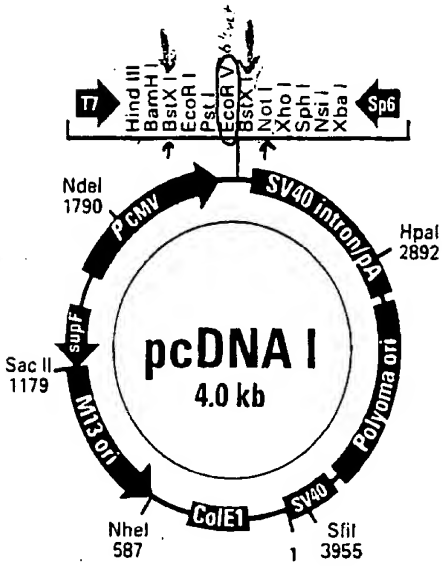
hSR-BI

11	12	13
		14

X163 Prepare blunt-cut pcDNA1 for reversing orientation of clone #76 mSR-BI

Purpose: Reverse orientation of clone #76 which appears to be full-length mSR-BI

Approach: Cut mSR-BI out of clone #76 with HindIII/NotI, chew ends to blunt it, ligate into blunt & CIPed pcDNA1



Cut pcDNA1 with EcoRV which is a blunt cutter

DNA	H_2O	10X BSA	10X NEB Buff 2	EcoRV 20,000 u/ml
~~15.5 µl (= 20 µg)~~ pcDNA1 (1.29 µg/µl) 10/29/92	14.5 µl	4 µl	4 µl	2 µl

Incubate 37°C 12:50 pm - 12:50 pm (Thurs)

← after 3 hrs

Add 60 µl ddH_2O, extract w/ 100 µl phenol:chloroform:isoamyl alcohol 25:24:1
Add 10 µl 3M NaOAc pH-5, add 250 µl 100% EtOH, Freeze -20°C 1 hr
Spin 10 min 4°C

[X163] cont'd

4 blunt-cut pcDNA1 (cont'd) - CIP

redissolve in 90µl 10 mM Tris·Cl (pH8)
remove 2µl & save as unCIPed

To remainder add 10µl 10X CIP dephosph. buffer and 8 Units of CIP:

for blunt 1U/2 pmoles, estimate 20µg = 16 pmoles
so 8 Units CIP

Incubate 15 min at 37°C 3:35 - 3:50

Then ~~add 8 Units more of~~ incubate 45 min at 55°C. 3:50 - 4:35

Kill the CIP

Add SDS & EDTA (pH8.0) to final 0.5% & 5mM, respect. (5µl 10% SDS, 1µl 0.5M EDTA)
Mix well
Add proteinase K to final 100µg/ml (10µl of 10 mg/ml)
Incubate 30 min 56°C. (4:50 - 5:20 pm)

Cool the rxn to r.t.
Extract once w/ phenol, once w/ phenol:chloroform
Add 1/10 vol 3M NaOAc pH ~~3.0~~ 5.0 (10µl)
Mix well add 2.5 vol EtOH (250µl)
Mix & store @ -20°C ~~o/n~~ 2 1/2 hrs
Centrifuge
Wash pellet w/ 70% EtOH
Redissolve in TE

Ran overnight on 1% Nusieve (should have been Seaplaque) Agarose gel. Band was wary, but cut it out anyway

X164 Prepare #76 mSR-BI cDNA for reinserting into pcDNA1 in correct orientation

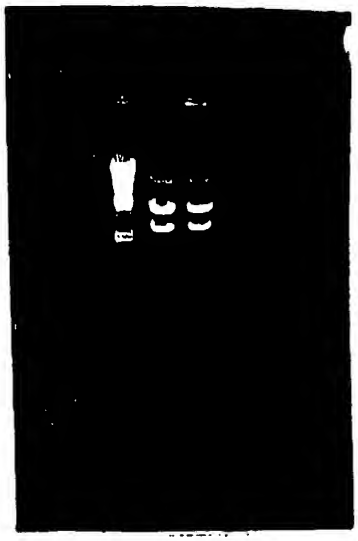
Purpose: Cut out #76 insert to be reinserted in sense orientation. #76 plasmid appears to be full-length mSR-BI but is in the incorrect orientation in pcDNA1.

Digest #76 plasmid:

	DNA	H_2O	10X BSA	10X buffer	HindIII	NotI
(1)	#76 (0.85 µg/µl) 24µl (= 20µg)	46µl	10	10 (NotI)	5	5
(2)	#76 24µl	46µl	10	10µl (NEB 2)	5	5

5:45pm 8:15pm 37°C

Remove 2µl from each and analyze on 1% Seakem GTG Agarose TAE gel



To the remainder add 10µl 3M NaAc + 250µl 100% EtOH
Store at -20°C for 15 min
Spin 10 min 4°C
Wash w/ 70% EtOH
Resuspend in TE (30µl)
Add 4µl 10X blue juice

To blunt ends, add 5 µl 10X T4 DNA Polymerase Buffer
+ 2 µl of dNTP mix (25mM) for final 1mM
+ 7 µl H_2O = 50 µl rxn ~~37° 30 min~~
+ 1 µl T4 DNA Polymerase 3U/µl 37° 30 min (11:30am - 12 noon)

Add 4 µl 0.25M EDTA
Add 55 µl Phenol/Chloroform/Isoamyl Alcohol (25:24:1)
Shake, spin, save aq. layer

To aq. phase
Add 10
1/10 V 5M NaCl (= 5 µl)
Shake add 5 µl of 1X linear polyacryl
add 150 µl 100% EtOH
Chill @ -80°C 10'
Spin 10', 4°C
Wash pellet w/ 70% EtOH
Speed vac dry

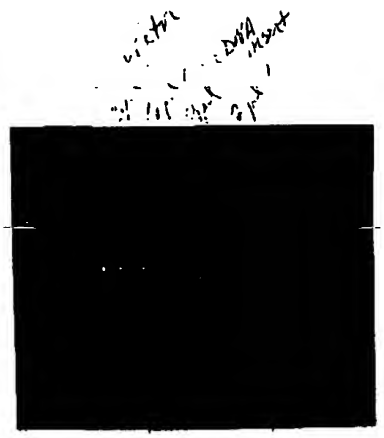
Resuspend pellet in 36 µl TE
Run 2 µl on mini-gel

Add blue juice & run on 1% TAE Seaplaque agarose gel in cold room at 20 V (med strength app)
5:45pm – 11:00 am
Cut out insert (~2.3 kb) & ligate into vector (see following page)

XII-5

Ligation Rxns.

1) Run blunt vector + blunt insert on 1% GTG Seakem gel to quantitate relative amounts



Estimate that 3µl vector = 3µl cDNA

Want ~ twice as much insert as vector in 10µl rxn so use 4µl vector and 6µl insert

2) Heat tubes to 70°C for 10-15 min to melt agarose
3) Combine aliquots of melted gel slices in tubes prewarmed to 70°C

tube	vector CIPed	vector not CIPed	blunt cDNA insert	H_2O	incubate 5-10 min 37°C	2X Ligation rxn mix (ice-cold)
1	4µl	–	–	6µl	✓	10µl
2	–	–	6µl	4µl	✓	↓
3	4µl	–	6µl	–	✓	↓
4	–	4µl	–	6µl	✓	↓

2X Lig. rxn mix:
10µl 1M Tris Cl pH 7.5
1µl 1M $MgCl_2$
2µl 1M DTT
1.2µl 86 mM ATP
86.3µl H_2O
100µl

Take 38µl & add 4µl T4 DNA Ligase

Incubate 48hr 12-16°C (7pm Sat - 12pm Mon)

X165 cont'd

Transformation of Ligations into MC1061/P3 E. coli

1. Melt the agarose/ligation mixtures at 70°C for 10-15 min.
2. Meanwhile thaw competent MC1061/P3 (from J. Ashkenas)

transform				Results # colonies
1	Lig #1	CIPed vector	5µl	56
2	Lig #2	insert (cDNA) only	5µl	28
3	Lig #3	CIPed vector + insert	5µl	30
4	Lig #4	UnCIPed vector	5µl	>500
5	TE only		5µl	47
6	pcDNA1 - 100ng/µl		1µl	>500

3. Add 5µl of melted agarose ligation mixtures or control also Mix by gentle shaking
Store on ice 30 min 12:45-1:15
4. Transfer to circulating water bath at 42°C
Incubate exactly 90 sec. DO NOT SHAKE TUBES
5. Rapidly transfer to ice bath. Allow cells to chill 1-2 min.
6. Add 450µl LB medium warm to 37° & transfer to shaking incubator at 37°C Incubate 1hr
1:25-2:25
7. Plate onto LB Amp/Tet (15µg/ml / 8µg/ml) plates
100µl/100mm plate
Incubate 37°C overnight

Results: Insert did not ligate into CIPed vector. Vector is CIPed well. Try blunting cDNA again and Geneclean both it and vector.

VII.G

Geneclean - vector + cDNA insert (#76)

	volume	2.5V NaI	glassmilk
insert -	150µl	375µl	10µl
vector -	150µl	375µl	10µl

Since ligation did not work between vector & cDNA, need to make sure ends of cDNA are blunt

Remove protruding 3' termini from cDNA insert

Combine: 15µl of genecleaned mSR-BI cDNA insert
1µl of 1:1:1:1 mix of dNTPs (25mM of each) ⇒ final 1.25mM
2µl of NEB Buffer 2 10X (final: 10mM Tris 10mM $MgCl_2$, 50mM NaCl 1mM DTT pH 7.9)
2.0 ~~µl H2O~~ µl T4 DNA Polymerase (3 U/µl)
Incubate 15 min 12°C

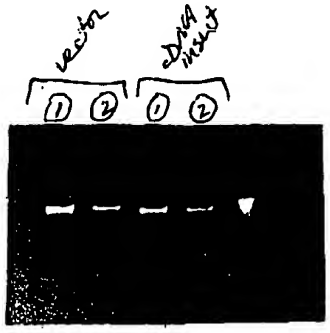
Inactivate polymerase by heating to 75°C for 10 min (11-11:10 am)

Run vector + cDNA insert on minigel to quantitate amts

1µl + 2µl TE ① → 1µl → + 2µl TE ②

run 2µl + 2µl 2x blue juice = 0.67

run 2µl + 2µl 2x blue juice = 0.22



For a 1 vector : 2 insert molar ratio use equal ng of vector & insert
Looks like vector stock = 1.5x cDNA

Ligate cDNA into vector

Ligation	blunt pcDNA1 CIPed	msR-B1 blunt cDNA	H_2O	BRL 5X T4 DNA Lig buffer	BRL T4 DNA Ligase (1U/μl)
1	3μl	-	4.5μl	2μl	0.5μl
2	3μl	4.5μl	-	2μl	0.5μl
3	1μl unCIPed vector	-	6.5μl	2μl	0.5μl

Mix well, spin & incubate 12-16°C (~18°C) 12:15pm - 10:30 Fri (~45hrs)

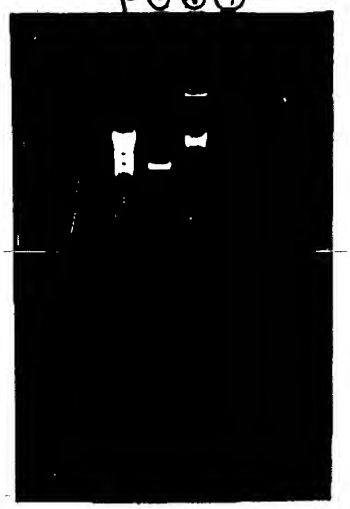
Transform MC1061/P3

transf		amt	results # colonies
1	Lig #1	5μl	123
2	Lig #2	5μl	32
3	Lig #3	5μl	33
4	TE only	5μl	
5	pcDNA1 388ng/μl	4μl	>1000

transformation worked but either result alone doesn't make sense

1) Add 5μl of above DNA to thawed competent MC1061/P3. Mix by gentle shaking. Store 30' ice (10:40-11:10)
2) Transfer to circulating water bath at 42°C. Incubate exactly 90 sec.
3) Rapidly transfer to ice bath. Allow cells to chill 1-2 min.
   Add 450μl LB Warm to 37°C & transfer to shaking incubator at 37°C.
4) Plate onto LB Amp/Tet (150μg/ml / 8μg/ml) 100μl/100mm dish. Incubate 37°C O/N.

Ran ligation rxns on gel to check



Lig

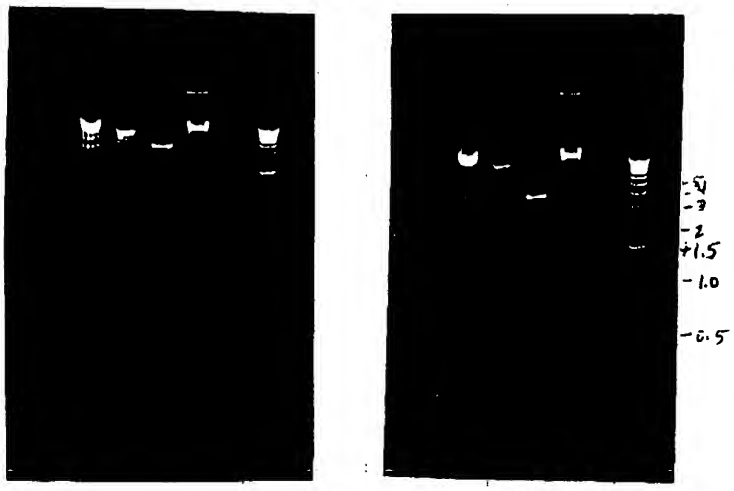
#1 - one piece but 6Kb!? Should be 4Kb. Must have cut the wrong vector? Redo preparation of blunt-cut cipcal pcDNA1 (see below)

#2 - some ligations appear to have worked possibly cDNA - cDNA worked best. Next time cut way back on amt of cDNA : vector.

#3 - can't see any DNA

Rerun with 1 Kb standards

Results:
blunt cut pcDNA1 is 4Kb as it should be
λHindIII stds are junk



X167

Redo Ligations

Ligation	blunt pcDNA1 CIPed	msR-BI blunt cDNA	H_2O	BRL 5X T4 DNA Lig buffer	BRL T4 DNA Ligase (1U/μl)
1	1μl	-	14	4μl	1μl
2	1μl	1μl	13	4	1
3	1μl	1μl of 1:5	13	4	1
4	1μl of 1:5	1μl of 1:5	13	4	1
5	1μl of 1:5	-	14	4	1

Mix well, spin & incubate 12-16°C (submerged) O.N. (5pm - 2pm)

Transform			Kb Sizes # colonies
1	Lig #1	- 5μl	1/50μl comp MC1061/P3 cells 68
2	2	- 5μl	136
3	3	- 5μl	130
4	4	- 5μl	605
5	5	- 5μl	19 + 30 in pink large colony
6	TE	- 5μl	5
7	pcDNA1	- 1μl	>2000

Ligate 30' 2:30 3pm
Shock 42°C 90 sec
Cool ice 1-2 min
Add 400μl LB & shake 37°C 45'
Plate 100μl LB A/T (#1-4 my plates, #5-7 Xpress plates)

PCR colonies

Purpose: Determine if colonies from transformation plate #2 contain mAR-BI in correct orientation

Set A (determine if insert present): T7 + ST6 primers
Set B (determine orientation): T7 + 0544/5

For 55 rxns of 20µl each

110 µl 10X PCR buffer (QG)
27.5 µl T7 primer @ 1 pmol/µl (VH)
425.7 µl H_2O
2.2 µl dATP
2.2 µl dCTP
2.2 µl dGTP
2.2 µl dTTP
5.5 µl Taq polymerase

(A) 412.5 µl → + 137.5 µl ST6 @ 1 pmol/µl

412.5 µl → (B) + 2 µl stock 0544/5 (unknown conc.) + 135.5 µl H_2O

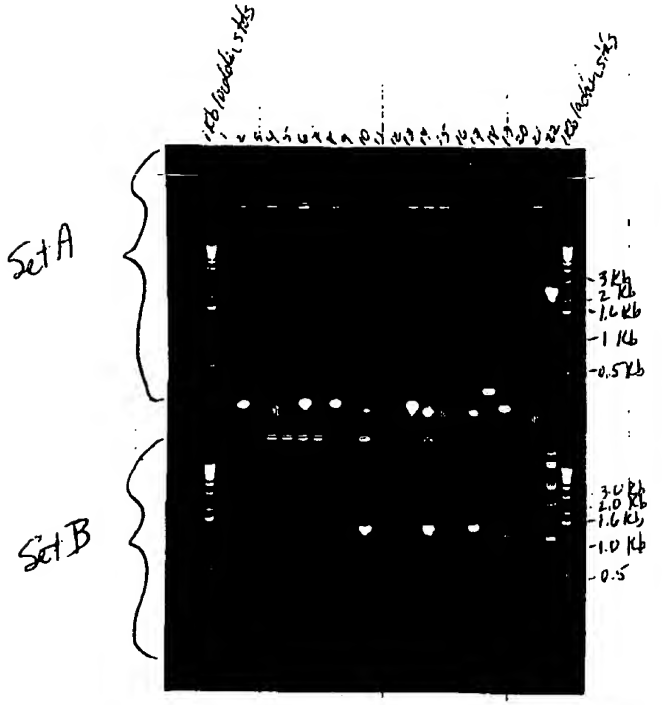
Pick 20 colonies
#21 neg control colony from transformation plate #1
#22 control #36 pure plasmid in SN BI in pcDNA1 backward

From 20 random colonies

10 will probably be vector only (no insert) - background
10 will " contain cDNA
of which 5 will be backwards
and (5 will be correct orientation

Run gel on PCR products

Run 1% GTG-Agarose. 1X TAE



Results: Only the positive control pure plasmid #76 (pCDNA/4 mSRBI) worked with the T7 + 5'Pst primers

However, a band of ~1.3 Kb was present in #10, #14 & #17. The expected size is ~1.35 Kb for mSR BI in sense orientation. Hopefully these are real.

Plasmid midiprep

preps: #2, #10, #14, #17 colonies from ligation/transform plate 2

Day 1

1. Pick a single colony into 25 mls LB Amp/Tet and grow overnight shaking 37°C.

Day 2

2. Take 400 µl, put into freezer vial, add 100 µl glycerol and freeze at -150C.
3. Transfer half to Falcon 2059 15 ml tube on ice.
4. Spin in SS-34, 9000 rpm, 2 min 4C. Discard supe, add remaining bacteria and respin.
5. Dry pellet as much as possible.
6. Resuspend pellet in 500 µl ice cold solution I by vigorous vortexing.
7. Add 1 ml fresh solution II (0.2 N NaOH, 1% SDS)
   for 10 ml: 1 ml 2 N NaOH
   0.5 ml 20% SDS
   8.5 mls ddH20
   Swirl gently until clear. Do not vortex. Leave on ice 10 min.
8. Add750 µl solution III (ice-cold). Close tube and mix contents by shaking vigorously several times. Store on ice 5'. A flocculent white precipitate should form.
9. Centrifuge 15', 4°C, 9000 rpm.
10. Recover supe and add equal volume of phenol:chloroform. Mix by vortexing.
11. Spin 9000 rpm, 5'. Recover aq. phase.
12. Add 2 volumes, ethanol r.t., vortex, let stand 5'.
    Spin in SS-34 rotor for 15' 9,000 rpm.
13. Carefully aspirate off all liquid (want it dry) and rinse once in cold 70% EtOH. Aspirate again getting any extraneous drops. Airdry 10' minutes.
14. Redissolve in 100 µl TE plus DNAse-free RNAse (20 µg/ml). Vortex briefly. Incubate 37C 0.5 - 2 hr. Transfer to ~~sterile~~ eppendorf.

~~15. Quantitate by dilutions onto EtBr plate.~~

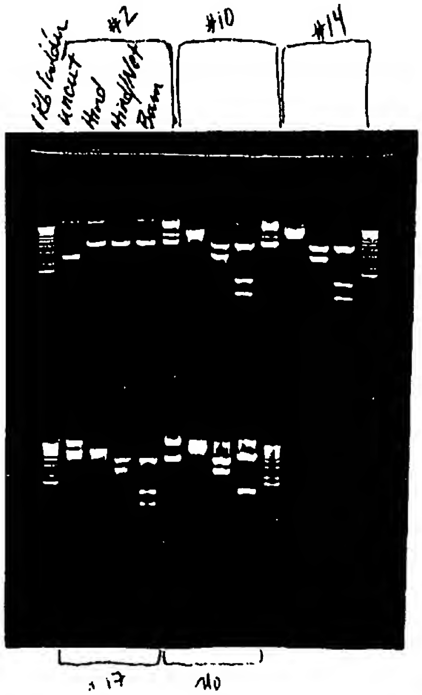
[redacted]

Digests

Digest	DNA	10X AcBSA	NEB Buffer 10X	H_2O	+ enzyme
1	colony #2 2μl	2μl	#2 2μl	14μl	
2			↓	13	Hind III
3				13	HindIII, Not I
#4			Change	13	Bam HI
5	colony 10				
6					
7					
8					
9	colony 14				
10					
11					
12					
13	colony 17				
14					
15					
16					
17	#76 mse BI (backwards)				
18					
19					
20					

Incubate 37°
12:45 - 3:45

Run on 1% Seakem GTG TAE gel



Results: Neg control #2 looks like pcDNA1 only. #10 #14 #17 all look same with insert size ~2.5 Kb. Not identical to #76 mse BI (backwards). Everything looks good! I think all three #10, #14, #17 are all in correct direction.
~~See next page~~

X168

DEAE dextran transfections of COS M6 cells

materials:

1. 35 mm dishes
2. DMEM with 10% FBS
3. Chloroquine (40 mM in CMF PBS, sterile filtered)
4. DNA
5. CMF PBS
6. DEAE-dextran (10 mg/ml in CMF PBS (autoclaved))
7. DMSO
8. cPBS
9. sterile tips

method:

day 0 (set up cells)

Set COS M6 cells in 100 mm dishes

(Set 1 confluent T75 into 2.25 100 mm dishes; or 1.5 x 10^6 cells/dish in 10 ml DMEM with 10% FBS

day 1 (transfect)

1. In sterile polypropylene tubes prepare for each dish add (in order):
   a) add CMF PBS to 1.9 ml
   b) DNA - 5 µg/dish
   c) 100 µl of 10 mg/ml DEAE-dextran, vortex well (7 seconds)

tube	# plates	DNA		CMF PBS	10 mg/ml DEAE-dextran
1	1	pcDNAI (1.29 mg/ml)	15µg 3.9ml	1.9 ml	100µl
2	3	pmSR-BI (2.1×10^11)	30µl	5.7 ml	300µl
3					
4					

2. Rinse cells with 10 mls CMF PBS (37C). Aspirate PBS.
3. Add transfection cocktail from step 1 and distribute evenly. Incubate 30 minutes, distributing liquid every 10 min. 1:30 - 2:00
4. Add 8 ml DMEM 10% FBS + 80 µM chloroquine and incubate 37C 2.5 hrs. 4:30
5. Aspirate off medium and replace with 5 ml 10% DMSO in DMEM 10% FBS for 2.5 min.
6. Aspirate off and wash once with 10 mls cPBS.
7. Refeed with 10 ml warm DMEM 10% FBS/dish. Incubate overnight.

Lipoprotein 4°C Binding Assay

materials:
1. 6-well dishes
2. DMEM with 10% FBS
3. iodinated and unlabeled lipoprotein
4. Tris-HCl wash buffer
5. Tris-HCl BSA wash buffer
6. 0.1 N NaOH
7. 10 x 75 tubes

method:

day 0: Set up cells

Set transfected COS in 6-well dishes at 1 x 10^6 cells/well in 3 ml/well DMEM + 10% FBS + 1 mM Nabutyrate.

day 1: Binding assay

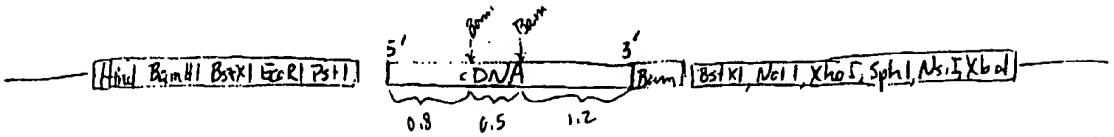
1. Cool cells down on ice for 30 minutes.
2. Refeed cells labeled ligand 1 ml/well (Hams + HEPES + ~~10% FBS~~ 5% NCLPDS)
   2 Hot - ^{125}I-lipoprotein: 10 mg/ml ^{125}I AcLDL #62 (0.55 mg/ml)
   ~~1 Hot + cold~~: competitors (a) 400 mg/ml
   Incubate 2 hrs 4°C (in cold room) on shaker.
3. Wash cells (1 ml each)
   3X fast with ~~BSA~~ wash buffer
   ~~2X 5 min with BSA wash buffer~~
   2X fast with ~~Tris wash buffer~~
4. Add 1.0 ml 0.1 N NaOH. Leave 20 min r.t. on shaker
5. Remove 50 µl to 10 x 75 tube and freeze for Lowry.
6. Count 500 µl of rest of sample.
7. Count 10 µl of medium + label for specific activity.

AcLDL #55 4.6 mg/ml
LDL #16B 39
HDL #3 31.2
VLDL #5 20.7

300 µl ^{125}I-AcLDL #62 + 17 mls Hams + 5% NCLPDS + 10 mM Hepes

4 mls + 348 µl AcLDL #55
2 mls + 70.5 µl LDL #16B
2 mls + 75.6 µl HDL #3
2 mls + 38.6 µl VLDL #3

mSR-BI in correct orientation:



mSR-BI (#76) in incorrect orientation: